The X gene of hepatitis B virus shows a high level stimulation of the Rous sarcoma virus long terminal repeat in the methylotropic yeast, *Pichia pastoris*

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Abstract In order to study the transactivational property of the X gene in the methylotropic yeast *Pichia pastoris*, a Rous sarcoma virus-chloramphenicol acetyltransferase (RSV-CAT) cassette was co-transformed and integrated into the host yeast strain as a reporter which showed an overwhelming CAT activity. Immunoprecipitation of the yeast cell extracts with an X-specific monoclonal antibody, however, showed a low level expression of the X gene. Therefore besides a *trans*-effect of the X protein, the enhanced reporter activity could be a manifestation of a *cis*-effect of the X gene sequences also. Therefore, unlike the transactivation studies with X gene in animal cells where limited functional activity is observed, *P. pastoris* appears to be an excellent system to study *cis*- and *trans*-aspects of gene regulation by the X gene.

Key words: Transactivation by HBV X gene product; Heterologous gene expression; Rous sarcoma virus long terminal repeat; *Pichia pastoris*

1. Introduction

The hepatitis B virus (HBV) is the infective agent for the widespread liver disease in humans known as hepatitis B. The small 3.2 kbp DNA genome of HBV has at least four open reading frames called S, C, P and X. During the natural course of HBV infection, the X gene expresses a polypeptide (HBx) of 154 residues that is implicated in HBV-mediated hepatocellular carcinoma [1,2].

The X gene is the smallest of four open reading frames and is expressed during the natural course of HBV infection [3]. Under certain conditions, the X gene product or HBx can have oncogenic potential that may be relevant for HBV-associated liver carcinogenesis [4], but until now the mechanism for cell transformation by HBx has been unclear. Investigations suggest that HBx is a pleiotropic transactivator (for reviews [5,6]). The pleiotropic nature of HBx may be attributed to the diverse properties associated with it. It displays ATPase and protein kinase-like activities, interacts with a number of host-cell factors, and more importantly can modulate cellular signal-transduction pathways. HBx is a transcriptional activator of not only the HBV genome but also a wide range of other viral and cellular promoters. It is known to activate target genes that incorporate *cis*-elements for some common *trans*-factors like AP-1, AP-2, C/EBP and NFκB. However, there is no evidence for a direct interaction between HBx and these transcription factors. Characterisation of protein domains that are important for its various activities have allowed identification of at least two regions of HBx that are essential for its transactivation function [7]. Extensive mutational analysis has shown that other regions in the molecule were dispensable for this function. The ability of X5 to transactivate the RSV-LTR was similar to the wild-type values. Our desire to express a transactivation competent mutant of X in yeast was further enhanced by the fact that this protein expressed in *Escherichia coli* was biologically inactive and showed minimal functional activity in animal cells [7] rendering further usage very difficult. *Pichia pastoris* on the other hand has been an excellent system for expressing biologically functional heterologous proteins in a soluble form.

P. pastoris has proved to be superior to *S. cerevisiae* for the expression of certain proteins [8,9] and has now been developed as an efficient host for heterologous gene expression using the promoter from the gene coding for alcohol oxidase (AOX1) [10,11]. Alcohol oxidase is the first enzyme in the methanol utilisation pathway and is induced by methanol [12] and constitutes about 20–30% of total cellular protein in methanol-grown cells. Regulation of heterologous gene expression by methanol has been shown to be simple, easy to scale up and cost-effective for industrial fermentation [9]. The functional components of the *P. pastoris* yeast expression system are the transfer vector and the host. The *his*$^-$ host strain can metabolise methanol as its sole carbon source by the action of the AOX1 gene product. The promoter for this gene induces a high level of tightly controlled gene expression. Recombination of the transfer vector into the yeast genome provides for auxotrophic selection by providing the ability of the transformed yeast to grow on plates lacking histidine. The vector is biodesigned to replace the structural component of the AOX1 gene with the recombinant gene of interest upon recombination with the yeast genome. Proper recombination of the transfer vector results in a *his*$^+$ and *mut*$^-$ (lacking the ability to utilise methanol efficiently) phenotype. Providing the recombinants with methanol as a sole carbon source induces expression of the recombinant protein.

In this study, we report the expression of a transactivation competent mutant (X5) of X gene of HBV in *P. pastoris*. An RSV-CAT cassette was co-transformed and integrated into the yeast *P. pastoris* as a reporter for transactivation by X. The expressed X protein in *P. pastoris* is highly bioactive since overwhelming CAT activity can be seen compared to the cor-

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Abbreviations: HBV, hepatitis B virus; AOX1 and 2, alcohol oxidase 1 and 2; CAT, chloramphenicol acetyltransferase; RSV-LTR, Rous sarcoma virus long terminal repeat; HBx, hepatitis B virus X protein; PAGE, polyacrylamide gel electrophoresis; bp, base-pair; kbp, kilobase-pair

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responding untransformed host or host with either the RSV-CAT cassette or X alone.

2. Materials and methods

2.1. Culture media

MD (minimal dextrose) medium is 1.34% yeast nitrogen base (YNB), 1% dextrose, and 4×10^{-5}% biotin. MM (minimal methanol) medium is 1.34% YNB, 0.5% methanol, and 4×10^{-5}% biotin. YPD and *E. coli* culture media were prepared using standard protocols.

2.2. Host strains and recombinant plasmids

The *P. pastoris* strain GS115 (*his4*$^-$), a derivative of NRRLY-11430 (SC-5) that has been previously described [12] was used in all yeast experiments. The *P. pastoris* expression plasmid used for all the constructs was pAO815 [13] (8.12 kbp) commercially available from Invitrogen, Carlsbad, CA, USA. The three plasmid constructs used in this work were pAO815-X-CAT (13.12 kbp), pAO815-X (8.12 kbp) and pAO815-CAT (12.71) as shown in Fig. 1 (A, B and C respectively). The X5 gene open reading frame [7] was cloned at the *Eco*RI site, to create pAO815-X. The unique *Bam*HI site in the original plasmid was used to insert the RSV-CAT reporter cassette [14] to create pAO815-CAT. pAO815-X-CAT was constructed by cloning the RSV-CAT cassette at the *Bam*HI site of pAO815-X5.

2.3. Transformation, screening and selection of expressing transformants

Vectors pAO815-X-CAT, pAO815-CAT and pAO815-X were digested with *Bgl*II prior to transformation, generating a fragment capable of displacing the chromosomal AOX1 gene by double crossover recombination (transplacement) [15]. *P. pastoris* host GS115 (*his4*$^-$) was transformed using the LiCl method as described before [11].

Transformants were visible after 3–4 days. These AOX1$^-$ transformants had the *mut*^{s} phenotype that showed slow growth on minimal media containing methanol as the sole carbon and energy source due to replacement of the AOX1 structural gene with the foreign gene. The reason AOX1 transformants grow at all on methanol is due to low-level expression of a second functional alcohol oxidase (AOX2)-encoding gene [15]. In initial screening, the His4 gene was used as a selectable marker, with about 20% of the transformants showing the *mut*^{s} phenotype.

Twenty five *mut*^{s} transformants were grown in a 5 ml glucose medium for 48 h after which they were switched to the methanol induction medium as described previously [11]. The cells were harvested by centrifugation at 3000 rpm for 5 min and suspended in alkaline lysis buffer pH 10.5 containing 50 mM NaOH/2 mM EDTA/1 mM PMSF/2% SDS/10% glycerol. The suspension was boiled for 5 min and adjusted to pH 7.5 with 1 M HCl. Total cellular protein samples were electrophoresed in a 15% polyacrylamide gel with SDS as described previously [11]. Expressing clones were selected by Western blot analysis (Fig. 1D) using the primary antibody against HBx as described previously [7].

2.4. CAT assay

Functional analysis of X5 was performed on RSV-CAT in induced cell lysates from transformants and appropriate control cells. CAT activity was measured as described [14]. Quantitative assay was performed by measuring the radioactivity of the substrate and product spots using liquid scintillation counting.

3. Results

3.1. Expression of X

To express X in *P. pastoris* we used the vectors pAO815-X and pAO815-X-CAT. The X gene [5] was inserted between the

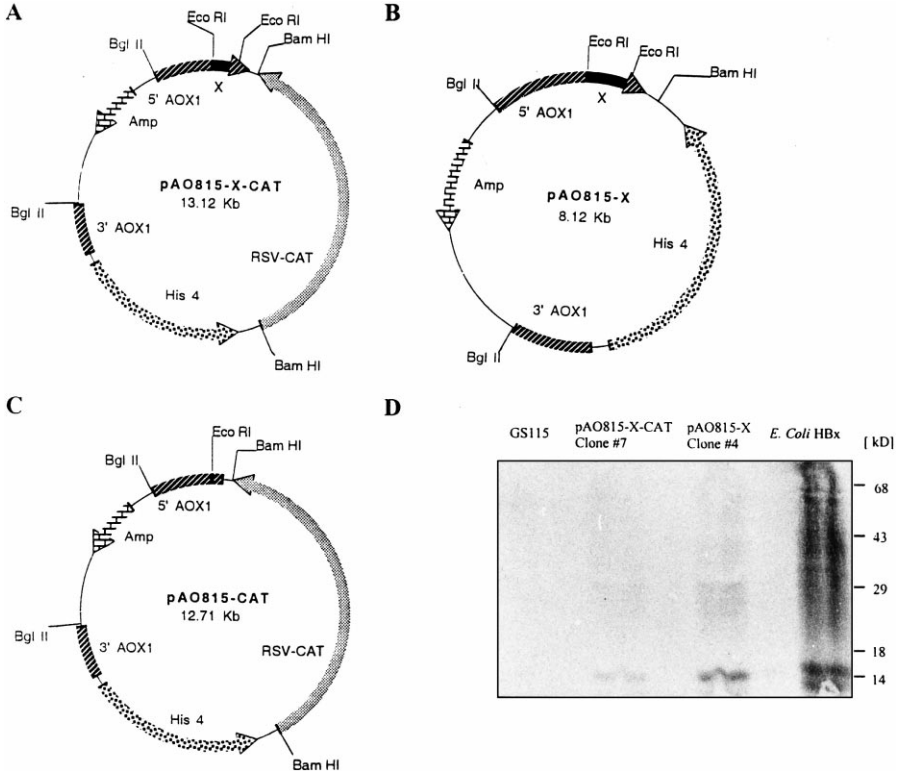

Fig. 1. Pichia pastoris expression constructs. Three expression constructs containing both the X gene and the RSV-CAT expression cassette (A) or the X gene alone (B) or the RSV-CAT expression cassette alone (C) were made using the pAO815 vector. All constructs contain genes for *his4* selection, ampicillin resistance (Amp), 5′ and 3′ alcohol oxidase 1 (AOX1) regions for integration into the AOX1 locus of the genome. D shows the Western blot analysis of the expressed HBx protein in transformants obtained by vectors pAO815-X-CAT and pAO815-X. GS115 is the untransformed host. The *E. coli* expressed X protein (16 kDa) was used as a size marker and a positive control on the Western blot. Protein HBx expressed in the *P. pastoris* transformants corresponds to 14 kDa since the insert was a transactivation competent mutant (X5) of X gene of HBV.

promoter and transcriptional terminator of the AOX1 gene. Induced transformants were lysed after three days and analysed by Western blots. Levels of expression of X were low but detectable on Western blots on pAO815-X and pAO815-X-CAT transformants (Fig. 1D). None of the pAO815-CAT transformants or untransformed host GS115 showed any bands on the Western blot.

3.2. Transactivation of RSV-LTR by X

To gain insight into the transactivation aspects of X protein, we made a construct pAO815-X-CAT having the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of RSV-LTR. This expression cassette was integrated into the *P. pastoris* host strain. The strategy to study transactivation in *P. pastoris* was to trigger the RSV-LTR by X. As can be seen in Fig. 2A, the expression of RSV-LTR was X-dependent and that all the five clones selected for the insertion of the RSV-CAT reporter, showed an overwhelming CAT activity (Fig. 2A, lanes 5–14). Cells harbouring either X gene (pAO815-X, Fig. 2A, lanes 1, 2) or RSV-CAT reporter (pAO815-CAT, Fig. 2A, lanes 3, 4) showed no CAT activity. The level of CAT activation by X was as high as 120-fold. For the present set of experiments cells were routinely harvested three days after induction. Further, the CAT activity in our experiments in the presence of X protein, was specific for the RSV-CAT reporter as can be seen in Fig. 2B. X gene (pAO815-X) was unable to induce any endogenous CAT activity even after three days of induction (Fig. 2B, lanes 1–8). Under identical experimental conditions however, pAO815-X-CAT was able to induce an overwhelming CAT response (lanes 9 and 10).

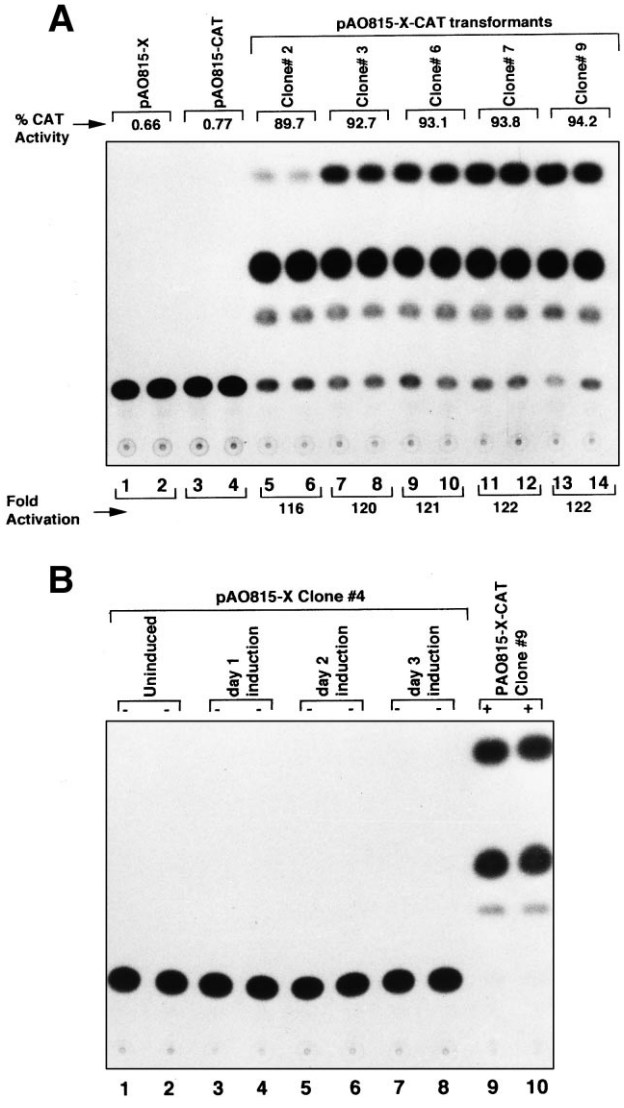

Fig. 2. Functional analysis of X on RSV-CAT. All clones were analysed in duplicate. (A) Lanes 1 and 2 show CAT activity for a transformant with X gene alone. Lanes 3 and 4 show CAT activity for a transformant with the RSV-LTR CAT construct alone. Lanes 5–14 show CAT activity for five selected transformants with pAO815-X-CAT. (B) Lanes 1 and 2 show CAT activity of an uninduced transformant with pAO815-X. Lanes 3–8 show the pAO815-X transformant 1, 2 and 3 days post-induction. Lanes 9 and 10 are a pAO815-X-CAT clone, 3 days post-induction.

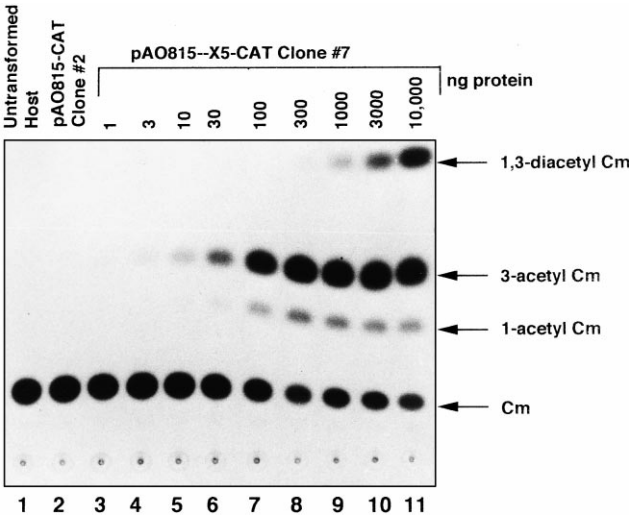

Fig. 3. Specificity of the CAT activity. Lane one shows CAT activity for the untransformed host. Lane 2 shows the activity for a transformant with CAT only. Samples 3–11 show a gradient of increasing ng protein level (crude lysate) of the pAO815-X-CAT transformant (clone #7). The three forms of acetylated chloramphenicol are marked as 1-acetyl chloramphenicol (Cm), 3-acetyl Cm and 1,3-diacetyl Cm.

3.3. Specific CAT activity as a function of protein concentration

A CAT activation analysis as a function of the amount of protein taken for the assay was performed. Results (Fig. 3) showed that as little as 3 ng of the crude extracts were able to show CAT activity (lane 4). The CAT activity increased with increasing concentration of protein (lanes 5–9). A concomitant increase in 1,3-diacetyl form of chloramphenicol is suggestive of the presence of highly active form of CAT enzyme in these extracts. It is also possible that more stable CAT enzyme expressed in yeast is more stable under the present experimental conditions.

4. Discussion

The data presented in this report suggest that biologically active transactivation competent HBx can be expressed in *P. pastoris* by utilising the AOX1 promoter based system. *P.*

pastoris can grow on methanol as the sole carbon and energy source as it contains a highly regulated methanol utilisation pathway [16,17]. The AOX1 promoter is tightly regulated and is used in this study to trigger HBx expression. HBx expressed in *P. pastoris* is in the soluble form and can be readily extracted by mechanical shearing of cells under non-denaturing conditions. Compared with expression in prokaryotes, yeast expression has the advantage of providing eukaryotic post-translational modifications. Expression in *P. pastoris* thus leads to a correctly folded highly bioactive product, as is the case with HBx. This transactivator protein has shown, in *P. pastoris* to be of great potential by the fact that it shows an overwhelming CAT response. This suggests that the yeast intracellular environment is more conducive to the folding/secondary modifications of HBx into its native state.

No difference in the levels of transactivation of RSV-LTR has been found when the wild-type X protein or its mutant X were transfected separately in HepG2 cells [7]. Since the N-terminal deletion (X5) in the X gene had no bearing on its transactivation function, we used this X recombinant in the present studies. We observed that the RSV-LTR could be stimulated to a highly significant level (up to 120-fold) by HBx. Under identical experimental conditions however, no stimulation of the reporter was observed in the absence of X suggesting the specificity of transactivation. As no background CAT activity was seen in the control samples. This suggested that none of the endogenous *P. pastoris* transcription factors were able to stimulate a heterologous viral promoter like RSV-LTR.

SDS-PAGE analysis of the yeast cell extracts from the X-harbouring cells apparently did not show induction of any endogenous genes (data not shown). This suggests that X protein may not be functioning as a universal transactivator in *P. pastoris*. Further, no increase in endogenous CAT-like activity was seen in yeast cells having X gene. Western blot analysis revealed that X protein could be expressed to a very low level in *P. pastoris*. Though this observation speaks in favour of a highly bioactive X protein, a '*cis*'-effect of the X-encoding sequence on the RSV-LTR can not be ruled out. It may be pertinent to mention here that the X-encoding region of the HBV genome also encompasses a strong enhancer element (Enhancer II) and promoter elements for nucleocapsid gene [18]. Thus, besides a *trans*-effect of the X protein on RSV-LTR, the core promoter and enhancer elements of HBV may be functional in *P. pastoris*. Transcription factors bound to these regulatory elements could exert a profound effect on the RSV-LTR leading to a significant increase in the reporter activity. It would be interesting to investigate further whether the increased CAT activity in *P. pastoris* is a manifestation of a '*trans*'- or '*cis*'-effect of the X gene or is a combined effect of the two. In either case, it would be interesting to work out the molecular mechanism of transactivation from the RSV-LTR in yeast cells. Initiation of eukaryotic transcription by polymerase II depends on a set of general transcription factors. These include TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH group of proteins. Initiation of transcription requires assembly of general transcription factors and RNA polymerase II on the DNA template to form a preinitiation complex [19]. Activation of transcription involves interaction of other transactivators with this preinitiation complex which could be involved in protein-DNA or protein-protein interaction. Recent reports suggest that HBx can directly interact with some of these general transcription factors, like TFIIB, TFIID and TFIIH [20–22]. These factors are reported to be present in yeast.

From the present study, we have been able to show a very high level of HBx-mediated transactivation. It would be interesting to investigate the molecular mechanism of interaction of these general factors with HBx in *P. pastoris*.

HBx alone is able to transactivate the RSV-LTR in yeast. This implies that (a) recruitment of the transcription process can take place on the regulatory sequences of a mammalian virus and (b) the viral X protein is able to interact with yeast transcription factor/factors in the transcription initiation complex and activate the process of transcription.

In fact in the recent past, a number of yeast proteins (XAP1 [23], XAP3 [24], XAPC7 [25], XIP [26], UVDDV [27] and p^{55sen} [28] have been cloned using the yeast two-hybrid system. These proteins show very diverse functions suggesting the promiscuous nature of HBx. The most alarming of all is UVDDV since it is considered important in the repair process of damaged DNA. HBx may be interacting with such proteins in its natural host and may influence the DNA repair process and transcription. It would be interesting to investigate if one of these or a new yeast protein mediates the X-dependent transactivation of RSV-LTR in *P. pastoris*. It is unlikely that an X-like factor may exist in yeast, because the X-responsive promoters, RSV-LTR, do not show any background reporter activity when *P. pastoris* is grown on methanol.

In this study a viral factor (HBx) is activating a viral LTR in a lower eukaryote like yeast. For the first time, a viral gene has been expressed to study viral gene regulation in a methylotrophic yeast expression system.

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